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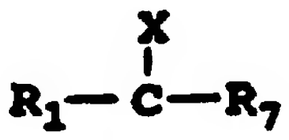
(54) Title: FLUORESCENT PEPTIDES

(57) Abstract

The invention provides biologically active compounds of formula (I) where R_1 is a light-emitting moiety and R_7 is a peptide of between 2 and 200 amino acids. X is selected from the group including =O, =S, -OH, =C=O, =NH, -H, -OR, -NR, -R, $-R_3R_4$, wherein each R, R_4 and R_3, independently, is H or a C_1-C_6 branched or unbranched, substituted or unsubstituted, alkyl. The compounds are both biologically active and optically detectable. The peptide is chemically attached to the light-emitting moiety at an amino acid position which is not involved in binding to the peptide's receptor. In this way, the peptide's affinity for its receptor is not significantly decreased, and the compound retains high biological activity and can be easily detected using standard optical means.



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FLUORESCENT PEPTIDES

Background

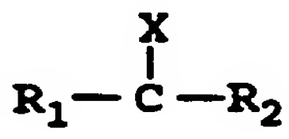
This invention relates to peptide-based compounds having light-emitting moieties.

Detectably labelled peptides provide useful reagents for monitoring peptide, cytokine, drug, and hormone receptors at the cellular level. Typically, the labelled peptide is placed in contact with a tissue or cell culture where it binds an available receptor. Once bound, the label is detected, allowing properties such as receptor distribution or receptor binding kinetics to be monitored.

Summary of the Invention

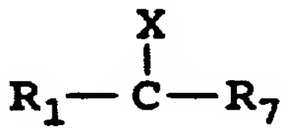
The invention provides a compound containing a peptide and a light-emitting moiety which is both biologically active and optically detectable. The peptide is chemically attached to the light-emitting moiety at an amino acid position which is not involved in binding to the peptide's receptor. In this way, the peptide's affinity for its receptor is not significantly decreased, and the compound retains high biological activity and can be easily detected using standard optical means.

In general, in one aspect, the invention provides a biologically active compound of the formula:



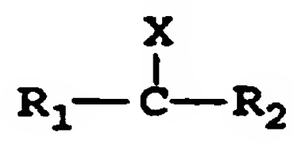
where R_1 is a light-emitting moiety; R_2 is a peptide of between 2 and 200 amino acids which is not neurotensin; and X is selected from the group consisting of =O, =S, -OH, =C=O, =NH, -H, -OR, -NR, -R, $-R_3R_4$, wherein each R, R_4, and R_3, independently, is H or a C1-C6 branched or

unbranched, substituted or unsubstituted, alkyl. In another aspect, the invention provides a biologically active compound of the formula:



where R_1 is a light-emitting moiety and R_7 is a peptide of between 2 and 200 amino acids bound to C by a binding moiety selected from the group including the residues Ala, Arg, Asn, Asp, Cys, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. Preferably, in this case, the peptide R_7 is not neurotensin. In both cases, X is selected from the group including =O, =S, -OH, =C=O, =NH, -H, -OR, -NR, -R, $-R_3R_4$, wherein each R, R_4 and R_3, independently, is H or a C1-C6 branched or unbranched, substituted or unsubstituted, alkyl.

In yet another aspect, the invention provides a biologically active compound of the formula:



where R_1 is a light-emitting moiety, R_2 is a peptide of between 2 and 200 amino acids, and X is selected from the group consisting of =O, -OH, =C=O, =NH, -H, -OR, -NR, -R, $-R_3R_4$, where each R, R_4 and R_3, independently, is H or a C1-C6 branched or unbranched, substituted or unsubstituted, alkyl. Preferably, in this case, the peptide R_2 is not neurotensin.

In preferred embodiments, both R_2 and R_7 are selected from the group including adrenocorticotrophic hormone, amylin, an amyloid beta-fragment, angiotensin,

an atrial natriuretic peptide, bombesin, bradykinin, cadherin, calcitonin, a casopmorphin, a morphiceptin, cholecystokinin, corticotropin-releasing factor, a dermorphin, dynorphin, an endorphin, endothelin, enkephalin, fibronectin, galanin, a gonadotropin-associated peptide, a gonadotropin-releasing peptides, a growth factor or growth factor-related peptide, gastrin, glucagon, growth hormone-releasing factor, somatostatin, a GTP-binding protein fragment, inhibin, insulin, interleukin, lutenizing hormone-releasing hormone, magainin, melanocyte-stimulating hormone, a morphiceptin, a neurokinin, a neuromedin, neuropeptide-Y, an opioid peptide, oxytocin, PACAP, pancreatic polypeptides, a parathyroid hormone, vasoactive intestinal polypeptide, Peptide YY, substance P, thyroid-releasing hormone, a toxin, vasopressin, and fragments, derivatives, and analogs thereof. Other possibilities for R_2 or R_7 are described in detail below. Most preferably, R_2 or R_7 are selected from the group consisting of angiotensin II, endothelin, galanin, and PACAP27.

In preferred embodiments, C is bonded to R_2 through an amino residue of an alpha carbon atom. Most preferably, R_1 is bound, through C, to a region of the R_2 peptide which is not involved in the peptide's biological activity, and the R_2 peptide binds to a human receptor.

In particular examples, R_2 is angiotensin and R_1 is bonded, through C, to the first amino-terminal amino acid residue of R_2 (e.g., Nα-Phe-). If R_2 is endothelin, then R_1 is bonded, through C, to the ninth amino acid residue of R_2 (e.g., the ϵ-amine group of Lys). If R_2 is PACAP 27, then R_1 is bonded, through C, to the thirteenth amino acid residue of R_2 (e.g., the ϵ-amine group of Lys). If R_2 is galanin then R_1 is bonded, through C, to the fifth amino acid residue of R_2 (e.g., the ϵ-amine group of Lys). In other preferred embodiments, R_1, the

light-emitting moiety, is selected from the group including fluorescein, FTC, Texas Red, phycoerythrin, rhodamine, carboxytetramethylrhodamine, DAPI, indopyra dyes, Cascade blue coumarin, NBD, Lucifer Yellow, propidium iodide, a porphyrin, and derivatives and analogs thereof. R_1 can be attached to C-X through a linking moiety selected from the group including indoacetamide, maleimide, isothyocyanate, succinimidyl ester, sulfonyl halide, aldehyde, glyoxal, hydrazine, and derivatives thereof.

In yet another aspect, the invention provides a method for generating a biologically active compounds described above. The method includes the steps of reacting R_1 and R_2 in an aqueous solution to form a compound mixture; contacting the compound mixture with a receptor for R_2; and isolating from the compound mixture a compound exhibiting biological activity in the presence of the R_2 receptor.

Preferably, the isolating step includes the steps of binding the compound to the R_2 receptor; releasing the compound from the binding complex; and isolating the biologically active complex.

In another aspect, the invention provides a method for generating a biologically active compound of claim 1 which includes the steps of reacting R_1 and R_2 in an aqueous solution to form a compound mixture; contacting the compound mixture with a receptor for R_2; isolating from the compound mixture a compound exhibiting biological activity in the presence of the R_2 receptor; determining biologically inactive regions of the isolated compound; and synthesizing one of the above-described compounds by chemically attaching the light-emitting moiety to an amino acid comprised in the biologically inactive region. The final compound is then formed by attaching all other amino acids to form R_2.

In still other aspects of the invention, the above-described compounds are used for labelling cell receptor sites, cell sorting, flow cytometry, and performing fluorimmunoassays. For example, cell receptor sites can be imaged by contacting candidate cell receptor sites with one of the above-described compounds, and then detecting the bound compounds as an indication of the cell receptor sites. Cell sorting can be performed by contacting a population of candidate cells with an above-described compound, and isolating cells bound to the compound. Flow cytometry can be performed by contacting a population of cells with an above-described compound, and detecting cells bearing receptors on their surfaces by detecting cells bound to the compound.

In all cases, by "biologically active" is meant the compound binds to a receptor having an affinity for the compound which is at least 0.25% of that of the corresponding unlabelled peptide. More preferably, the receptor affinity for the compound is at least 1.0% of that of the corresponding unlabelled peptide. Receptor affinity, in this case, can be determined either using known methods of fluorescence polarization for measuring K_d for the receptor-peptide interaction or by using methods involving competition binding with radioactively labeled peptides.

By "peptide", as used herein, is meant a chain of amino acids of any length. Included in this term are proteins and polypeptides.

By "compound mixture", as used herein, is meant a mixture of peptides which are bound to light-emitting moieties at different amino acid positions. Such a mixture may contain peptides of varying lengths and varying biological activities.

The invention has many advantages. In a general sense, peptide-containing compounds which retain their

biological activity after being labelled with light-emitting moieties have a wide variety of biological applications. Such compounds can be used effectively to identify, visualize, quantify, target, and select receptors on cells and tissues both in vitro and in vivo. These compounds obviate the use of more conventional labeled peptides, e.g., those attached to radioactive isotopes such as ^{125}I. Radiolabelled compounds are often toxic, environmentally hazardous, chemically unstable, and have, by nature of the isotopes' radioactive decay rates, relatively short lifetimes. In contrast, the light-emitting biologically active compounds of the invention are relatively safe, non-toxic, easily disposable, and may be synthesized without employing any special laboratory procedures. In addition, because most light-emitting moieties are relatively stable, the compounds can be stored for extensive periods of time without undergoing significant degradation.

The compounds of the invention offer many additional advantages when employed in biological experiments. For example, peptides labeled with light-emitting moieties emit optical signals following excitation, and thus may be monitored by eye or with the aid of conventional, easy-to-use optical detectors (e.g., conventional charge-coupled devices (CCDs) or light-sensitive cameras). This method of detection is, in general, relatively simple and cost effective compared to detection of radioactive particles.

In addition, unlike radiolabelled, enzymatic, and colorimetric compounds, the compounds of the invention do not have to be incubated with secondary labeled compounds for detection. This means that the compounds can be used to monitor dynamic biological phenomena, such as the kinetics associated with receptor binding, in real time.

Furthermore, selection of biologically active compounds using a receptor binding assay is advantageous because it allows generation of useful biological labels in a rapid and efficient manner. In contrast, radiolabelled peptides typically require distinct and time-intensive steps for first isolating the labeled peptides and then determining their biological activity.

Other advantages and features of the invention will become apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Fig. 1 is a schematic drawing of a compound containing a peptide and light-emitting moiety according to the invention;

Fig. 2 is a flow chart showing the general synthetic procedure used to produce the compounds of the invention;

Fig. 3 is a schematic drawing of the chemical structure of fluorescent angiotensin II according to the invention;

Fig. 4 is a schematic drawing of the chemical structure of fluorescent endothelin-1 according to the invention;

Fig. 5 is a schematic drawing of the chemical structure of fluorescent galanin according to the invention;

Fig. 6 is a schematic drawing of the chemical structure of fluorescent PACAP27 according to the invention;

Figs. 7A and 7B are, respectively, confocal laser scanning microscope (CLSM) and computer-generated images showing fresh, frozen rat kidney sections imaged with fluorescent angiotensin II;

Figs. 8A and 8B are, respectively, CLSM and computer-generated images showing fresh, frozen rat cerebellum sections imaged with fluorescent endothelin;

Figs. 9A and 9B are, respectively, CLSM and computer-generated images showing fresh, frozen rat cerebellum sections imaged with fluorescent PACAP; and

Figs. 10A and 10B are, respectively, CLSM and computer-generated images showing fresh, frozen rat brain septal regions imaged with fluorescent galanin.

Detailed Description

As discussed above and shown in Fig. 1, biologically active and light-emitting, peptide-based compounds 10 according to the invention are generated by attaching a light-emitting moiety 12, such as a fluorescent dye, through a -(C-X)- bond to a peptide moiety 14. The peptide moiety contains both a biologically active region 16 and one or more "inactive" regions 18 (i.e, regions that are not significantly involved in the peptide's biological role). In one particular example, the biologically active region binds to the peptide's associated receptor, while the biologically inactive regions do not significantly participate in the peptide/receptor binding process. In general, to retain a biological activity for a fluorescent peptide that is comparable to that of the native peptide, the light-emitting moiety is chemically attached at a site within one of the peptide's biologically inactive regions. In this manner, the light-emitting moiety does not sterically hinder or otherwise significantly affect the region involved in, for example, receptor binding, and the biological activity of the compound is thus maintained at a high level.

In general, because of their relatively small size, the compounds of the invention preferably exhibit a

1:1 molar ratio between the light-emitting and peptide moieties. The attachment of more than one light-emitting moiety per peptide may, in some cases, result in a loss of biological activity. The 1:1 molar ratio is also important for the quantification of receptors during labelling applications, as it allows measured fluorescence intensity to be directly translated into a measure of binding peptide moieties which, in turn, allows the number of receptors to be determined. In addition, multiple fluorophores attached to a single peptide may result in fluorescence quenching, i.e., a loss of emission intensity which sometimes occurs when fluorophores are present in very close proximity to each other.

In addition to having amino acids available for receptor binding, the peptide compounds of the invention also contain light-emitting moieties which retain optical properties similar to those of the unbound fluorophore. In this way, the compound, even when bound to its receptor, emits light following absorption of an incident optical field, and thus serves as a marker for that particular receptor.

The general synthesic method for generating light-emitting biologically active compounds of the invention is shown in Fig. 2. This method begins with the step 20 of incubating the peptide and fluorophore of choice to form a mixture of compounds. Incubation is performed under conditions which permit optimal peptide labelling (see below). Typically, a solution containing the peptide of choice (preferably at a concentration of 10^{-2}-10^{-4}M) is mixed with the light-emitting moiety in a highly basic solution (i.e., at a pH of 9.3-10.7), such as a carbonate buffer, in at least a 1:4 peptide:light emitting moiety molar ratio. The solution is mixed at room temperature for a time period of between about 24-48

hours, and is protected from light and shaken periodically. The resulting compound mixture includes biologically active and inactive whole peptides, cleaved fragments of peptides, and singly and multiply labelled peptides.

After covalent conjugation between the light-emitting moiety and peptide is allowed to occur, unbound fluorophore is removed (step 22). In general, this selection process can be performed using standard techniques, such a column chromatography or other analytical techniques known in the art. In a typical example, unreacted amounts of the free fluorophore are removed using a G-50 column equilibrated with phosphate-buffered saline (pH 7.4) and spun at 3000 rpm for a time period of between 5 and 20 minutes. The resultant eluent contains a mixture of labelled biologically active and inactive peptides.

This solution is then collected and subjected to a high-stringency pharmacological binding assay (step 24). In this assay, only biologically active compounds are bound to tissue receptors; inactive compounds are washed away. The assay is typically performed on tissue sections, receptor-coated columns, or membrane homogenates. In a typical example, an aliquot of the fluorescent peptide mixture is first dissolved in an aqueous solution (1:100) and incubated with an immobilized tissue sample containing high numbers of the peptide's receptor, e.g., rat neural membrane homogenates.

The selection process is designed to separate compounds exhibiting substantial biological activity from those relatively inactive compounds. If necessary, during the assay, binding of the biologically active compounds may be rapidly observed visually (from the sections), in a fluorometer (from precipitated membrane

homogenates), or by using more sensitive techniques such as fluorescence polarization spectroscopy.

The receptor-bound compounds are then removed from the tissue surface (step 26) and analyzed (step 28) to identify the site at which the fluorophore is attached (i.e., the site allowing fluorophore attachment without interference with receptor binding). Biologically active compounds bound to membrane receptors are separated from the remaining inactive fluorescent peptides in solution, either by centrifugation of membrane homogenates (typically at 3000 rpm for about 5 min. at 4°C) or, in the case of sections, by rapidly rinsing the sections in incubation buffer (3 x 1 minute) at 4°C. The membranes are then resuspended in binding buffer, with the biologically active compounds removed from the cell surface by incubation in a high salt/acid wash solution.

Once isolated, biologically active compounds are analyzed using known techniques, such as carboxypeptidase digestion and HPLC or amino acid sequencing, to identify the site of attachment between the light-emitting moiety and the peptide.

Once the attachment site is determined, the appropriate amino acid can be attached directly to the light-emitting moiety prior to synthesis of the peptide. This allows the compound to be synthesized in a highly purified form using standard techniques, such as solid-phase peptide synthesis (step 30). If desired, the resulting complex can be further purified (step 32), preferably using a column-based method such as HPLC, and then eluted. In this manner, large quantities of biologically active, labelled peptide compounds can be easily generated in an automated fashion.

In general, reactions between peptides and light-emitting moieties are carried out by modifying amino acid

functional groups, most typically a thiol or amine group, so that the moieties may be easily conjugated. Reactions for such modifications are described in the "Handbook of Fluorescent Probes and Research Chemicals - 5th Edition" by Richard P. Haugland (1992), the contents of which are incorporated herein by reference. In general, thiols react with alkylating groups (R'-Z) to yield relatively stable thiol ethers (R-S-R'), with the leaving group Z preferably being a halogen (e.g., Cl, Br, or I) or a similar moiety. The most common reagents for derivatization of thiols are haloacetyl derivatives. Reaction of these reagents with thiols proceeds rapidly at or below room temperature in the physiological pH range.

Light-emitting moieties may also be attached to amino acid amine groups. The conditions used to modify amine moieties of the desired peptide will depend on the class of amine (e.g., aromatic or aliphatic) and its basicity. Aliphatic amines, such as the α-amino group of lysine, are moderately basic and reactive with acylating reagents. The concentration of the free-base form of aliphatic amines below pH 8 is very low; thus, the kinetics of acylation reactions of amines by isothiocyanates, succinimidyl esters, and other reagents is strongly pH-dependent. Although amine acylation reactions should usually be carried out above pH 8.5, the acylation reagents degrade in the presence of water, with the rate increasing as the pH increases. The α-amino function of the amino terminus usually has a pK_a of ~ 7, thereby allowing it to be selectively modified by reaction at neutral pH.

In general, reactive groups on the light-emitting moiety, such as unsaturated alkyl groups, will react with the modified amino acid to form the compounds of the invention. The chemical structure of the light-emitting

moiety may affect the synthetic route used to synthesize the compound. It may be necessary, for example, to modify the light-emitting moiety so that it includes a reactive group prior to exposure to the desired peptide.

The above technique has been used to synthesize a biologically active compound containing somatostatin and a light-emitting moiety which is described in U.S.S.N. 08/475,751 entitled "Fluorescent Somatostatin", filed June 7, 1995, which is a continuation-in-part of U.S.S.N. 08/416,007, having the same name and filed April 4, 1995, the contents of which are incorporated by reference.

Figs. 3-6 show a number of different compounds made by the general method described above. Each compound features fluorescein, a light-emitting moiety, bound to an individual peptide at an amino acid position which preserves the compound's biological activity. In each of these compounds, the C-X bond is an acyl moiety. Table 1, below, lists the peptides and fluorescein-bound amino acids included in each of the compounds.

Table 1 - Biologically Active Light-Emitting Compounds

Figure	Peptide	Binding Amino Acid	Light-emitting moiety
3	Angiotensin II	1; Nα-Phe	fluorescein
4	Endothelin I	9; ϵ amine - Lys	fluorescein
5	Galanin	5; ϵ amine - Lys	fluorescein
6	PACAP27-NH_2	13; ϵ amine - Lys	fluorescein

The syntheses of the compounds of Table 1, along with biological experiments describing their applications, are described in detail in the Examples below.

In general, any peptide which exhibits an affinity for its corresponding receptor can be used to make a biologically active light-emitting compound of the invention. Peptides may be synthesized using techniques known in the art, extracted from natural systems, or obtained from commercial sources (e.g., Peninsula, Neosystems, Sigma, and BASF). Typically, the peptide is either purchased or synthesized using conventional solid-phase synthetic techniques. Preferably, the peptide is substantially pure, meaning that it is at least 60% by weight free from the other compounds with which it is naturally associated.

Preferred peptides are included in the group consisting of adrenocorticotrophic hormone, amylin, an amyloid beta-fragment, angiotensin, an atrial natriuretic peptide, bombesin, bradykinin, cadherin, calcitonin, a casopmorphin, a morphiceptin, cholecystokinin, corticotropin-releasing factor, a dermorphin, dynorphin, an endorphin, endothelin, enkephalin, fibronectin, galanin, a gonadotropin-associated peptide, a gonadotropin-releasing peptide, a growth factors or growth factor-related peptide, gastrin, glucagon, growth hormone-releasing factor, somatostatin, GTP-binding protein fragments, inhibin, insulin, interleukin, lutenizing hormone-releasing hormone, magainin, melanocyte-stimulating hormone, a morphiceptin, a neurokinin, a neuromedin, neuropeptide-Y, an opioid peptide, oxytocin, PACAP, a pancreatic polypeptide, parathyroid hormones, vasoactive intestinal polypeptide, Peptide YY, substance P, thyroid-releasing hormone, a toxin, vasopressin, and fragments, derivatives, and analogs thereof.

Peptides useful in the invention include those whose sequences differ from the wild-type peptide sequence by only conservative amino acid substitutions.

For example, one amino acid may be substituted for another with similar characteristics (e.g. valine for glycine, arginine for lysine) or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish the peptide's biological activity. Other useful modifications include those which increase peptide stability. For example, the peptide may contain one or more non-peptide bonds (which replace a corresponding peptide bond) or D-amino acids in the peptide sequence.

Other peptides which may be used include those described in the Peninsula Laboratories Inc. catalogue, 1992-1993; SIGMA-Peptides and Amino Acids catalogue, 1993-1994; and PIERCE, Catalog & Handbook, Life Science & Analytical Research Products, 1994, the contents of each of which are incorporated herein by reference.

The light-emitting moiety can be any moiety which emits an optical field following excitation. Preferably, the moiety is selected from the group consisting of fluorescein, FTC, Texas Red, phycoerythrin, rhodamine, carboxytetramethylrhodamine, DAPI, indopyra dyes, Cascade blue coumarins, NBD, Lucifer Yellow, propidium iodide derivatives thereof. Other light-emitting moieties used in labelling or other applications may be attached to the compound in place of the above. For example, suitable light-emitting moieties are described in Molecular Probes, Handbook of Fluorescent Probes and Research Chemicals, 1992-1994; and Richard P. Haugland et al., "Design and Application of Indicator Dyes", Noninvasive Techniques in Cell Biology: 1-20, Wiley-Liss Inc., (1990), the contents of each of which is incorporated herein by reference.

In general, these light-emitting moieties possess at least one side group capable of reacting with amino acids to form chemical bonds. Such side groups include

indoacetamide, maleimide, isothyocyanate, succinimidyl ester, sulfonyl halide, aldehyde, glyoxal and hydrazine derivatives. Amino acids including alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine may be labeled in this fashion.

In order to retain substantial biological activity and affinity for its receptors, the peptide moiety is attached to the light-emitting moiety by a -(CX)- bond. This bond can include groups such as C=O, C=S, CH(OH), C=C=O, C=NH, CH_2, CHOH, CHOR, CNR, CH-R, and C-R_3R_4, wherein each R, R_3, and R_4, independently, is H or a C1-C6 branched or unbranched, substituted or unsubstituted, alkyl.

Fluorescent compounds selected to have high biological activities have a number of uses. For most applications, the compound is first contacted with the sample of interest. The compound is then incubated with the cells or tissues of the sample for a select time period and allowed to interact with the receptor corresponding to the compound's peptide.

Once the peptide is bound to the desired receptor sites, the labelled sample is imaged using standard techniques known in the art. For example, conventional microscopy methods, such as fluorescence or confocal microscopy, may be used to optically excite and then detect emission from the labelled receptors. Other imaging techniques which may be used with the fluorescent peptides include atomic force microscopy, fluorescence polarization spectroscopy, and fluorimetry.

Using the above techniques, small-scale features in the cell which normally would be difficult to detect are observed. For example, these techniques allow

visualization of intracellular receptor sites. Moreover, labeled peptides participating in peptide-receptor interactions can be monitored to determine the location of receptors in cells or tissues, to allow quantification of receptors, to determine receptor affinity for various unknown ligands (drug screening), and to identify various populations of cells endowed with peptide receptors. Other applications include receptor sorting using FACS (fluorescence-associated cell sorting) and measurement of serum peptide levels using FIA (fluorescent immunoassays) either *in vivo* or *in vitro* for research or diagnostic purposes. In general, techniques which may utilize the compounds of the invention include, without limitation, flow cytometry, cell sorting (for example, to isolate populations of cells bearing a receptor of interest), tumor marking, competitive binding assays for drug screening, fluorescent immunoassays, and other *in vitro* experimental techniques involving compound labelling according to techniques known in the art.

The following Examples are used to more particularly point out the synthesis, selection methods, and use of fluorescent peptides having substantial biological activity.

Examples

Example 1 - Fluorescent Labelling of Angiotensin II, PACAP27, Galanin, and Endothelin

Twenty five μg of angiotensin II, PACAP27, and galanin, and 20 μg of endothelin purchased from Peninsula Laboratories Inc. (San Carlos, CA) were dissolved in separate solutions of 40 μl of 50mM bicarbonate buffer (NaH_2CO_3), pH 9.3, to a final dilution of 0.8 mM, or 10 μl of the same in the case of endothelin. NHS-fluorescein (N-hydroxy-succinimidyl ester) from Pierce Chemical Company (Rockford, Illinois) was dissolved in 100 μl of DMSO. 50-100 μl of this stock NHS-fluorescein (2.1 μmol)

was added while mixing the peptide solution. The sample was then placed on ice, incubated for one hour at pH 9.3, and then brought to pH 8 by the addition of 500mM Tris HCl. Incubation was allowed to proceed for the next 18 hours at 4°C.

Following incubation, unreacted fluorescein was removed using G-50 column chromatography (Pharmacia Biotech, Upsala, Sweden). Biologically active and inactive compounds were eluted with 0.1 M phosphate buffered saline (PBS) at pH 7.4 by spinning the column in a table-top centrifuge at 3000 rpm for 10 minutes. The eluent was dissolved and incubated with known quantities of rat renal membrane homogenates for angiotensin and endothelin, and rat brain homogenates for PACAP and galanin. The components of these assays were prepared according to standard techniques, with receptor binding monitored in a Beacon™ fluorescence polarization apparatus from Panvera Corp.

After allowing binding to occur over a 60-minute period for endothelin, angiotensin, and galanin, and 4-6 hours for PACAP27, membranes were precipitated from solution by centrifugation at 3000 rpm for 5 minutes at 4°C. Membranes were then resuspended in PBS and incubated with a solution equivalent to 0.5 M NaCl and 0.2 M acetic acid at pH 3.1 to strip surface-bound fluorescent peptides from their receptors on the cell surface. Using this method, the biologically active compounds were then collected for amino acid analysis.

The sites of attachment of fluorescein to angiotensin II, endothelin, PACAP-27, and galanin were confirmed to be at, respectively, the N-terminus N-alpha amino group of phenylalanine, the ninth amino acid (i.e., lysine) on the epsilon amino group, the thirteenth amino acid (lysine) on the epsilon amino group, and the fifth amino acid residue (lysine) on the epsilon amino group.

The molar ratio of fluorescein to peptide was confirmed to be 1:1 in each case.

Example 2. Fluorescent peptide labelling of peptide receptors on fresh frozen tissue sections

Female Sprague Dawley rats were sacrificed by decapitation, and their brains snap-frozen in isopentane at -40°C. Frozen sections of 20μm from the rat kidney and brain were cut on a cryostat at -20°C and thaw-mounted on gelatin-coated slides. The sections were dried in a desiccator overnight at -20°C.

Kidney sections were incubated in 500ml of 10mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl, 5 mM Na_2-EDTA, and 0.02% NaN_3 to remove any endogenous ligand. Sections were then incubated with fresh sodium phosphate buffer containing 0.9 nM angiotensin II, 0.4 M bacitracin, and 0.2% bovine serum albumin for 60 minutes at room temperature.

For fluorescent endothelin labeling, kidney sections were preincubated for 15 minutes in 20 mM HEPES buffer (pH 7.4) containing 135 mM NaCl and 2 mM $CaCl_2$. The sections were then incubated with 2 nM of fluorescent endothelin in 20 mM HEPES buffer (pH 7.4) containing 135 mM NaCl, 2 mM $CaCl_2$, 0.2% BSA, and 0.01% bacitracin for 2 hours at room temperature. Brain sections taken from the cerebellum region were incubated with 10-20 nM of fluorescent PACAP27 in 20 nM HEPES buffer (pH 7.4) in the above-described solution. For fluorescent galanin labeling, brain sections from the septal region of the forebrain were incubated with 2 nM of fluorescent galanin in 20 mM HEPES buffer (pH 7.4) containing 5 mM $MgCl_2$, 0.1% bacitracine, 1mM EDTA, 1% BSA for 45 minutes at room temperature.

After incubation, all rat tissue sections were transferred through 4 successive 1-minute washes of PBS buffer (pH 7.4) at 4°C. The slides were then rapidly

dried under a cold stream of air and examined using confocal laser scanning microscopy (CLSM) to observe the binding of the fluorescent peptides. Sections were initially examined on a Leica epifluorescence photomicroscope operating with a high-pressure 100 Watt mercury arc lamp; the appropriate dichroic filter combination for the excitation (485 nm) and emission (520 nm) wavelengths of fluorescein was employed to improve the signal-to-noise ratio of the fluorescence signal. Selected portions of the sections were further scanned using a Leica CLSM (St. Laurent, Quebec, Canada) composed of a Leica Diaplan inverted microscope equipped with an argon ion laser (488 nm) having an output power of 2-50 mW. All computer-generated images (i.e., Figs. 7B-10B) and processing operations were carried out using the Leica CLSM software package.

Referring now to Figs. 7-10, sections of rat kidney and brain sections incubated with fluorescent angiotensin, endothelin, PACAP, and galanin demonstrated discreet apple-green fluorescent labeling when monitored with the CLSM. Overall, the distribution pattern of fluorescent labeling conformed to that previously determined using radiolabelled analogs of the above peptides. In particular, the experiments indicated that only areas known to contain receptors were labelled while nearby areas devoid of receptors were completely unlabeled. This finding suggests that the compounds of the invention retain the peptides' high specificity for their receptors. In addition, all fluorescent peptide labeling was abolished by incubation with 100-fold excess of unlabeled peptide, further indicating that labeling with the compounds of the invention was selective for the peptides' receptors.

Figs. 7A and 7B are, respectively, CLSM and computer generated images of fluorescent angiotensin II

binding to 20 μm-thick sections from the rat kidney at 40X. In particular, the images illustrate that fluorescent angiotensin II effectively binds to rat kidney glomeruli, a region known to be enriched in angiotensin receptors. The labeling was confined to the juxtaglomerular apparatus observed in the lower right-hand corner of the image. The center of the glomeruli, shown in the upper left-hand corner of the micrograph, was unlabelled by the fluorescent compound. Incubation with 100-fold excess unlabeled angiotensin II resulted in an absence of labeling.

In the computer-generated image of Fig. 7B, white represents the highest level of binding, while red, yellow, green, and blue represent, respectively, decreasing levels of binding.

Figs. 8A and 8B are, respectively, CLSM and computer generated images of fluorescent endothelin binding to 20 μm-thick sections from the rat cerebellum, a region known to be enriched in endothelin receptors. Within the cerebellum, the labeling was confined to a Purkinje cell layer which, at higher powers, appeared concentrated throughout Purkinje cell bodies and large dendrites. The adjacent molecular layer, represented in the bottom portion of the photograph, was completely devoid of labeling. Incubation with 100-fold excess unlabeled endothelin resulted in an absence of labeling.

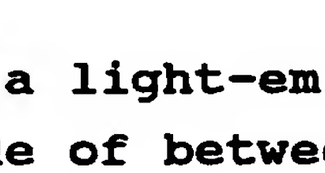
Figs. 9A and 9B are, respectively, CLSM and computer generated images of fluorescent PACAP binding to 20 μm-thick sections from the rat cerebellum at 40X. This region is known to be enriched in PACAP receptors. Within the cerebellum, the labeling was concentrated to the Purkinje cell layer; at higher powers labelling appeared concentrated throughout cell bodies and in fine proximal dendrites. Incubation with 100-fold excess unlabeled PACAP resulted in an absence of labeling.

Figs. 10A and 10B are, respectively, CLSM and computer generated images of fluorescent galanin binding to 20 μm-thick sections from the rat brain septal region at 40X. This region is known to contain galanin receptors. Within the septal area, the labeling was confined to numerous small fusiform neurons and proximal small dendrites, shown in the center and left-hand portions of the images. In the images, the cellular pattern of labeling was diffuse, whereas the dendritic labeling was of higher resolution.

Other embodiments are within the scope of the following claims.

What is claimed is:

1. A biologically active compound of the formula:

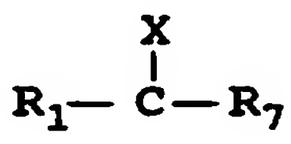


wherein R_1 is a light-emitting moiety;

R_2 is a peptide of between 2 and 200 amino acids and is not neurotensin; and

X is selected from the group consisting of =O, =S, -OH, =C=O, =NH, -H, -OR, -NR, -R, and $-R_3R_4$, wherein each R, R_3, and R_4, independently, is H or a C1-C6 branched or unbranched, substituted or unsubstituted, alkyl.

2. A biologically active compound of the formula:

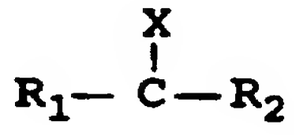


wherein R_1 is a light-emitting moiety;

R_7 is a peptide of between 2 and 200 amino acids bound to C by a binding moiety selected from the group consisting of the residues Ala, Arg, Asn, Asp, Cys, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val; and

X is selected from the group consisting of =O, =S, -OH, =C=O, =NH, -H, -OR, -NR, -R, $-R_3R_4$, wherein each R, R_3 and R_4, independently, is H or a C1-C6 branched or unbranched, substituted or unsubstituted, alkyl.

3. A biologically active compound of the formula:



wherein R_1 is a light-emitting moiety;

R_2 is a peptide of between 2 and 200 amino acids;

X is selected from the group consisting of =O, -OH, =C=O, =NH, -H, -OR, -NR, -R, $-R_3R_4$, wherein each R, R_4 and R_3, independently, is H or a C1-C6 branched or unbranched, substituted or unsubstituted, alkyl.

4. The compound of claim 1, 2, or 3, wherein C is bonded to R_2 through an amino residue of an alpha carbon atom.

5. The compound of claim 1, 2, or 3, wherein R_1 is bound, through C, to a region of said R_2 peptide which is not involved in said biological activity.

6. The compound of claim 1, 2, or 3, wherein R_2 is selected from the group consisting of adrenocorticotrophic hormone, amylin, an amyloid beta-fragment, angiotensin, an atrial natriuretic peptide, bombesin, bradykinin, cadherin, calcitonin, a casopmorphin, a morphiceptin, cholecystokinin, corticotropin-releasing factor, a dermorphin, dynorphin, an endorphin, endothelin, enkephalin, fibronectin, galanin, a gonadotropin-associated peptide, a gonadotropin-releasing peptides, a growth factor or growth factor-related peptide, gastrin, glucagon, growth hormone-releasing factor, somatostatin, a GTP-binding protein fragment, inhibin, insulin, interleukin, lutenizing hormone-releasing hormone, magainin, melanocyte-stimulating hormone, a morphiceptin, a

neurokinin, a neuromedin, neuropeptide-Y, an opioid peptide, oxytocin, PACAP, pancreatic polypeptides, a parathyroid hormone, vasoactive intestinal polypeptide, Peptide YY, substance P, thyroid-releasing hormone, a toxin, vasopressin, and fragments, derivatives, and analogs thereof.

7. The compound of claim 6, wherein R_2 is selected from the group consisting of angiotensin II, endothelin, galanin, and PACAP27.

8. The compound of claim 7, wherein R_2 is angiotensin and R_1 is bonded, through C, to Nα-Phe-.

9. The compound of claim 7, wherein R_2 is endothelin and R_1 is bonded, through C, to an ϵ-amine group of Lys.

10. The compound of claim 7, wherein R_2 is PACAP27 and R_1 is bonded, through C, to an ϵ-amine group of Lys.

11. The compound of claim 7, wherein R_2 is galanin and R_1 is bonded, through C, to an ϵ-amine group of Lys.

12. The compound of claim 1, wherein R_1 is selected from the group consisting of fluorescein, FTC, Texas Red, phycoerythrin, rhodamine, carboxytetramethylrhodamine, DAPI, indopyra dyes, Cascade blue coumarin, NBD, Lucifer Yellow, propidium iodide, a porphyrin, and derivatives and analogs thereof.

13. The compound of claim 12, wherein R_1 is attached to C-X through a linking moiety selected from

the group consisting of indoacetamide, maleimide, isothyocyanate, succinimidyl ester, sulfonyl halide, aldehyde, glyoxal, hydrazine, and derivatives thereof.

14. A method for generating a biologically active compound of claim 1, 2, or 3, comprising:

reacting R_1 and R_2 in an aqueous solution to form a compound mixture;

contacting said compound mixture with a receptor for R_2; and

isolating from said compound mixture a compound exhibiting biological activity in the presence of said R_2 receptor.

15. The method of claim 14, wherein said isolating step comprises

binding said compound to said R_2 receptor;

releasing said compound from said binding complex; and

isolating said biologically active complex.

16. A method for generating a biologically active peptide compound of claim 1, 2, or 3, comprising:

reacting R_1 and R_2 in an aqueous solution to form a compound mixture;

contacting said compound mixture with a receptor for R_2;

isolating from the compound mixture a compound exhibiting biological activity in the presence of said R_2 receptor;

determining the amino acid position at which said R_1 moiety is bound to said R_2 peptide; and

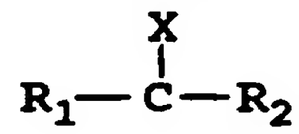
synthesizing said compound of claim 1 by chemically attaching said R_1 moiety to said amino acid of said R_2 peptide.

17. A method for imaging cell receptor sites comprising contacting candidate cell receptor sites with a compound of claim 1, 2, or 3 and detecting said bound compounds as an indication of said cell receptor sites.

18. A method for cell sorting comprising contacting a population of candidate cells with a compound of claim 1, 2, or 3 and isolating cells bound to said compound.

19. A method for flow cytometry comprising contacting a population of cells with a compound of claim 1, 2, or 3 and detecting cells bearing receptors on their surfaces by detecting cells bound to said compound.

20. A compound of the formula



wherein R_1 is a light-emitting moiety; R_2 is a peptide of between 2 and 200 amino acids; and X is selected from the group consisting of =O, =S, -OH, =C=O, =NH, -H,
-OR, -NR, -R, $-R_3R_4$, wherein each R, R_4, and R_3, independently, is H or a C1-C6 branched or unbranched, substituted or unsubstituted, alkyl;

wherein said compound is formed by:

reacting R_1 and R_2 in an aqueous solution to form a compound mixture;

contacting the compound mixture with a receptor for R_2;

isolating from the compound mixture a compound exhibiting biological activity in the presence of the R_2 receptor; determining biologically inactive regions of the isolated compound; and,

synthesizing said compound by chemically attaching R_1 to an amino acid comprised in said biologically inactive region to form a labeled amino acid, and by attaching other amino acids to said labeled amino acid to form said compound.

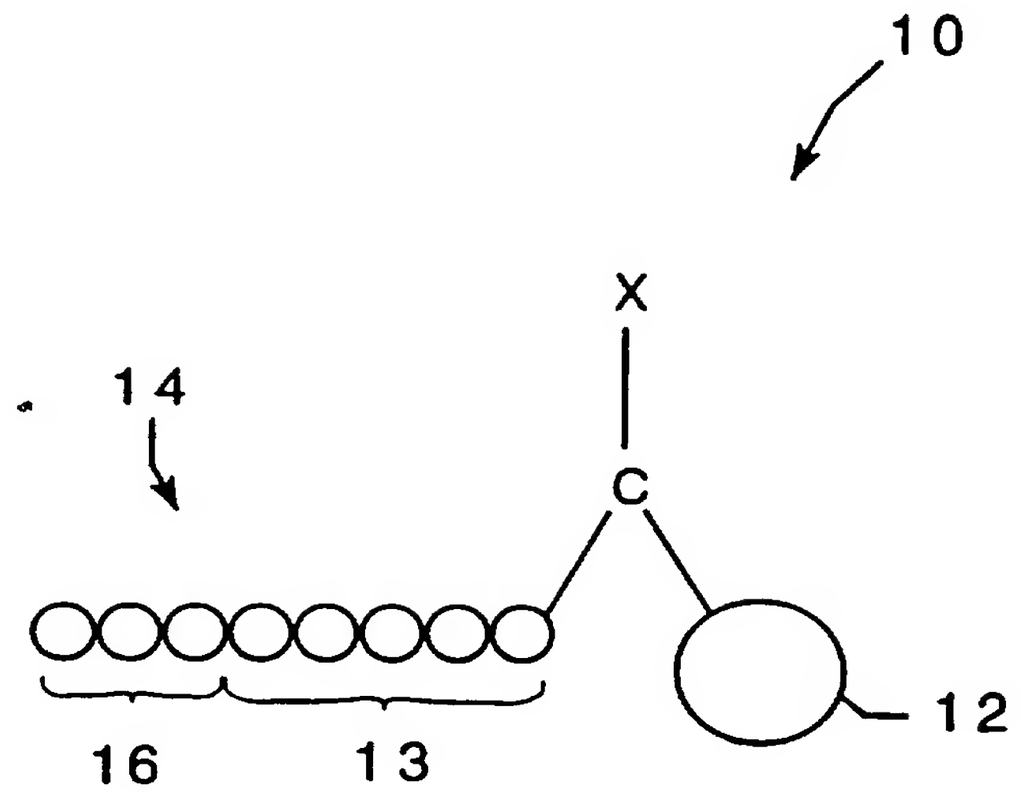


FIG. 1

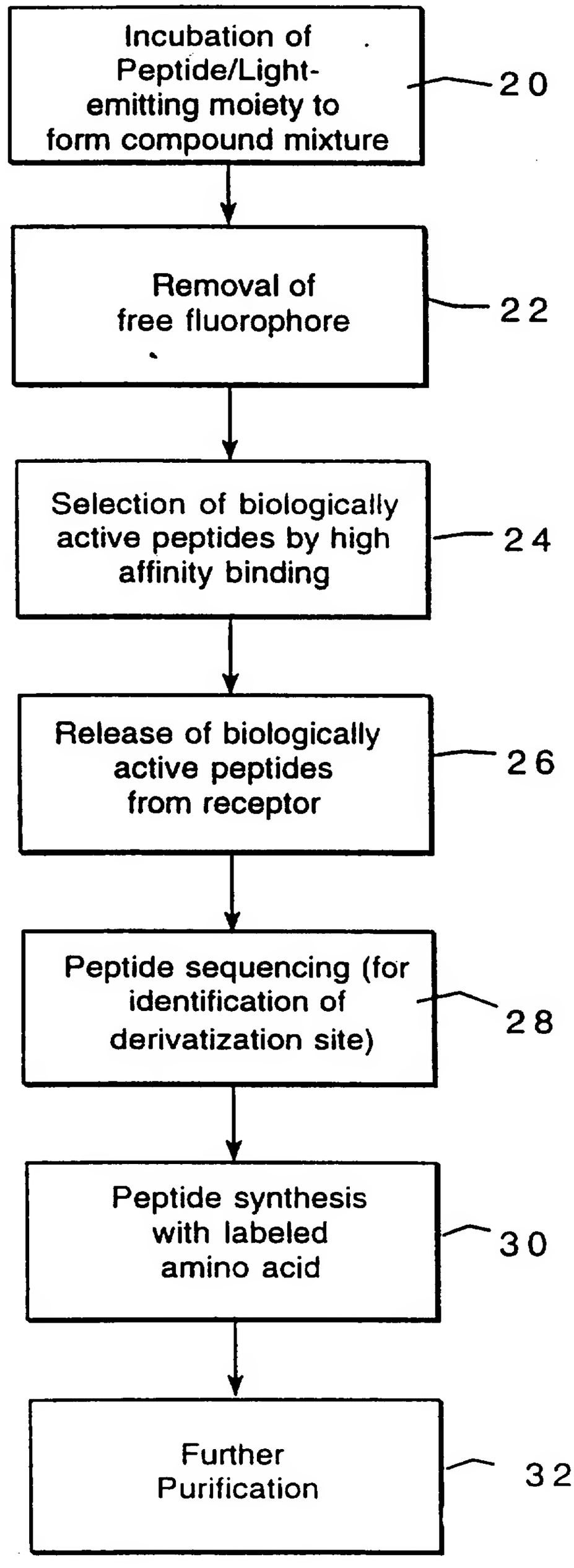

Incubation of Peptide/Light-emitting moiety to form compound mixture
20
Removal of free fluorophore
22
Selection of biologically active peptides by high affinity binding
24
Release of biologically active peptides from receptor
26
Peptide sequencing (for identification of derivatization site)
28
Peptide synthesis with labeled amino acid
30
Further Purification
32

FIG. 2

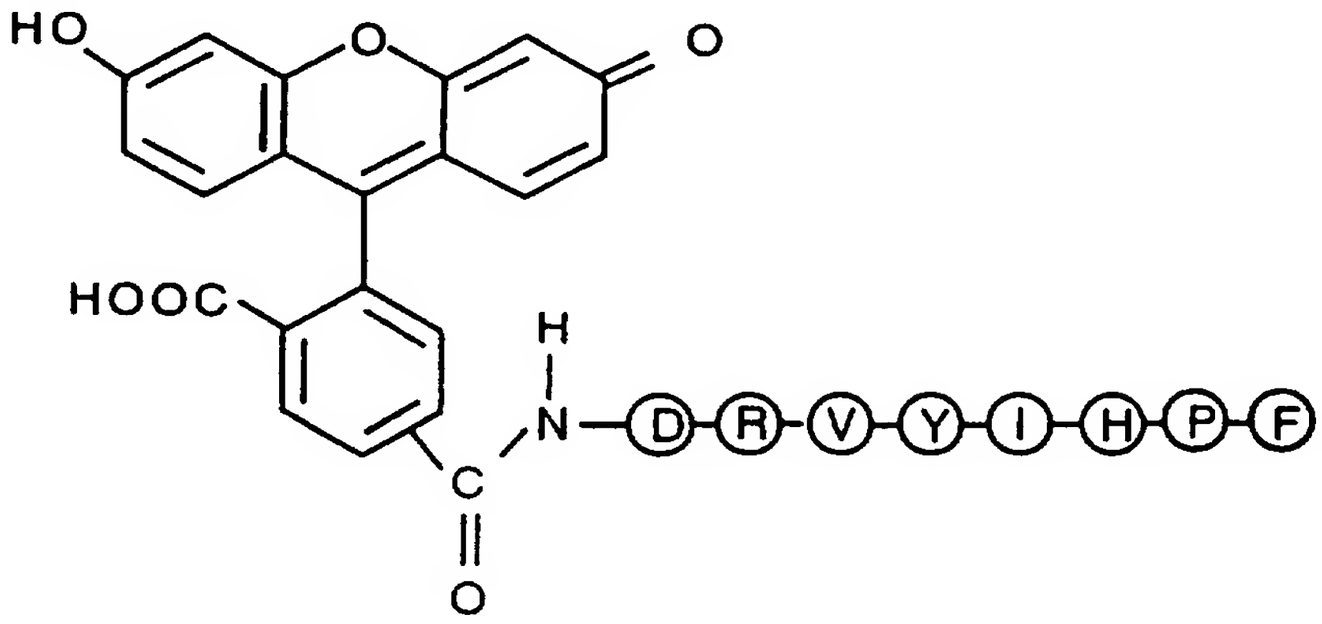

HO
O
O
HOOC
H
N
D R V Y I H P F
C
O

FIG. 3

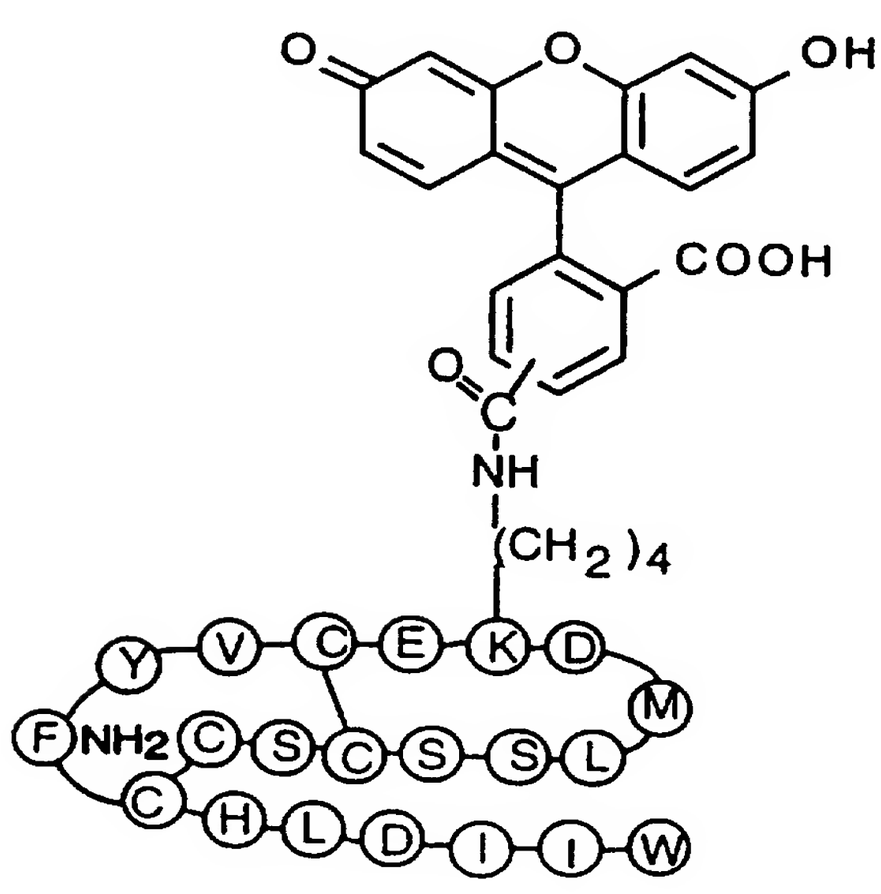

O
O
OH
COOH
O
C
NH
(CH2)4
Y V C E K D
M
F NH2 C S C S S L
C H L D I I W

FIG. 4

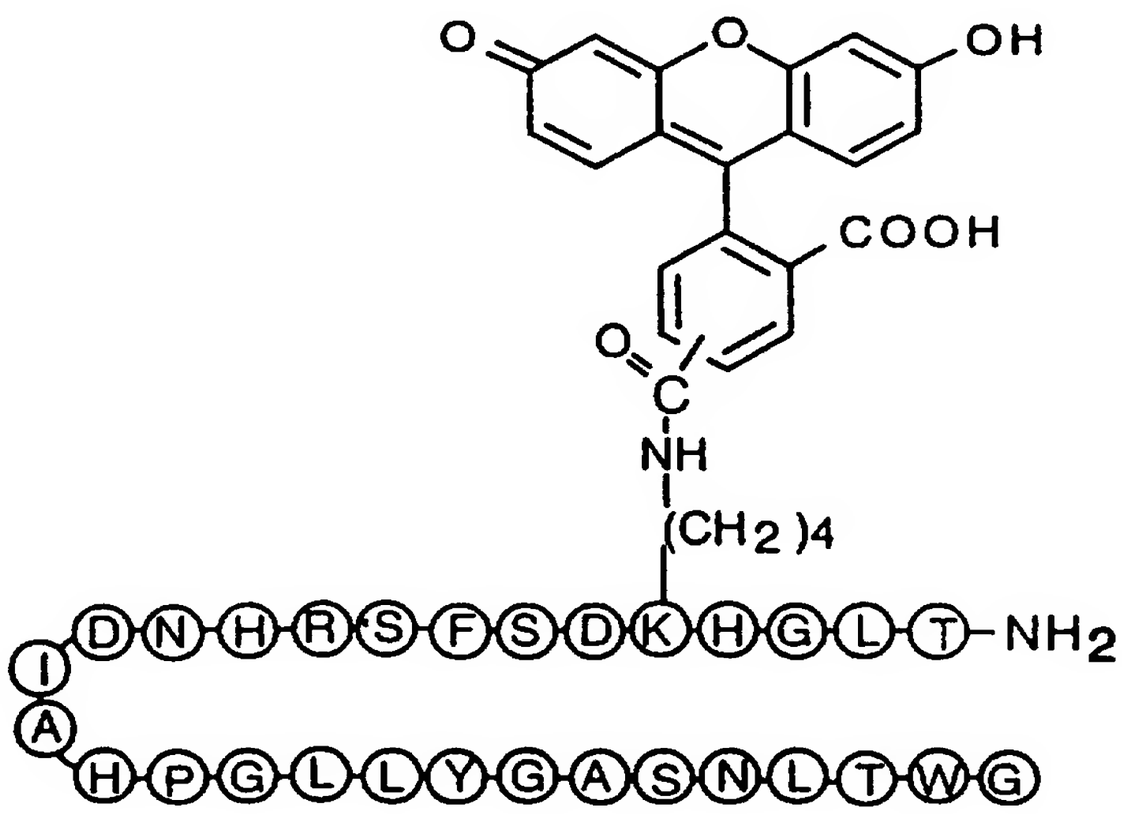

O
O
OH
COOH
O
C
NH
(CH2)4
D N H R S F S D K H G L T NH2
I
A
H P G L L Y G A S N L T W G

FIG. 5

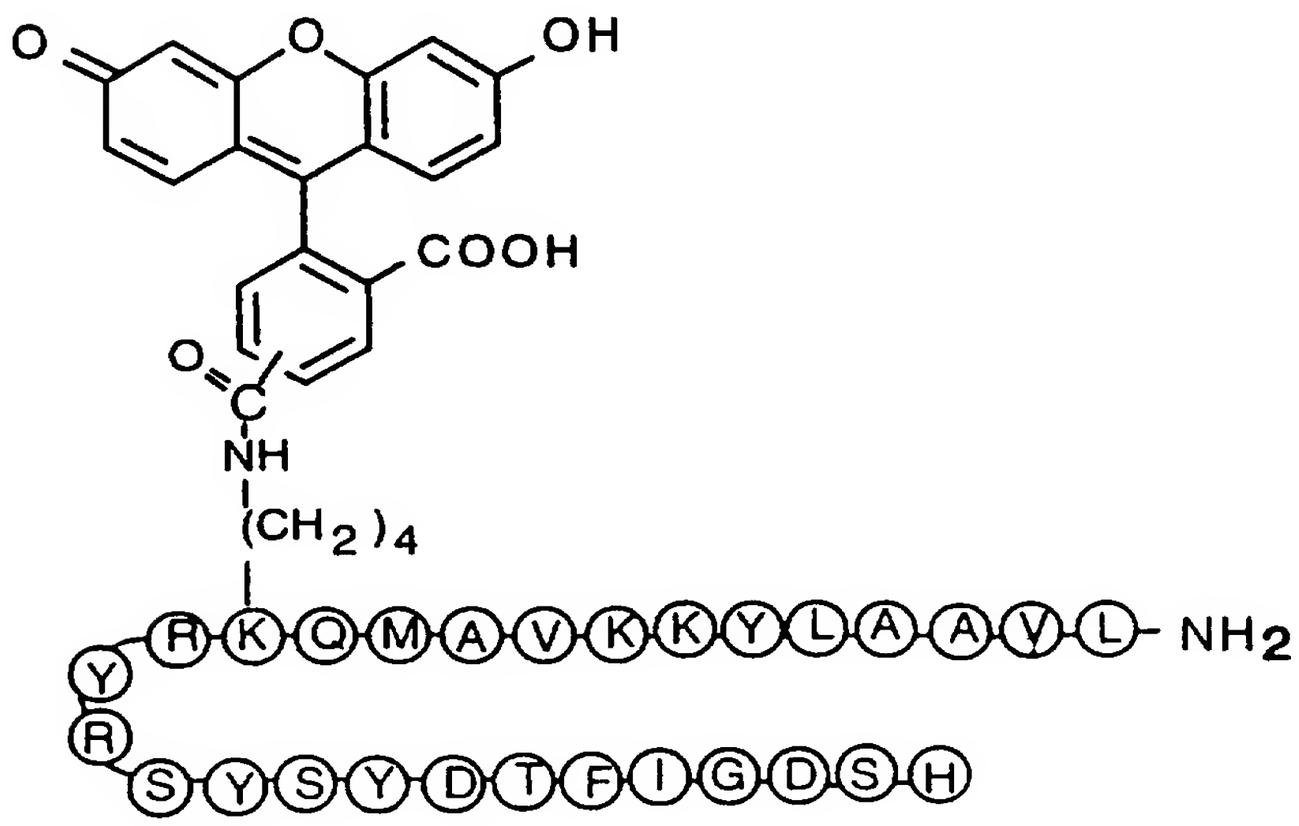

O
O
OH
COOH
O
C
NH
(CH2)4
R K Q M A V K K Y L A A V L NH2
Y
R
S Y S Y D T F I G D S H

FIG. 6

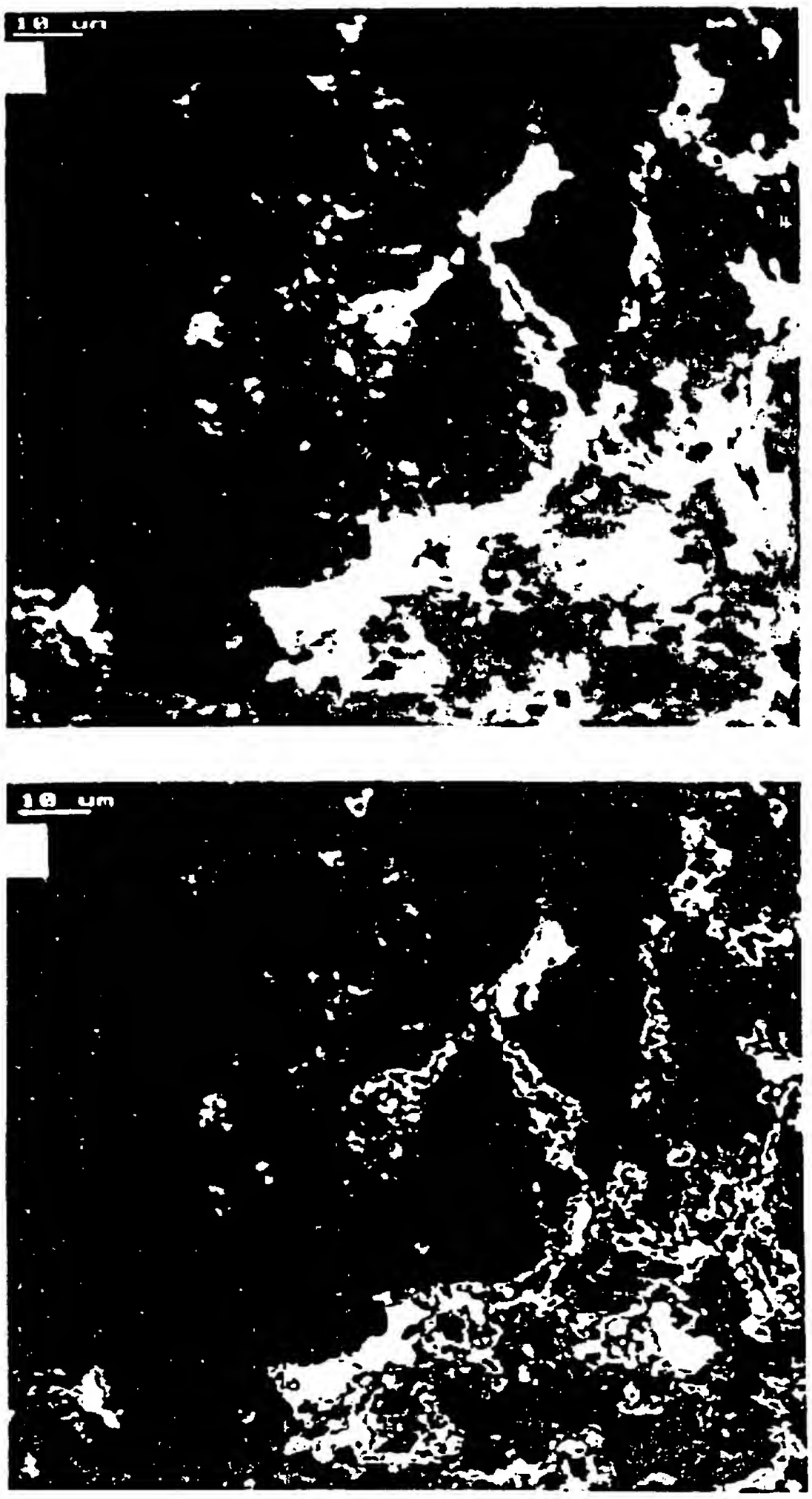


FIG. 7A

FIG. 7B

FIG. 8A

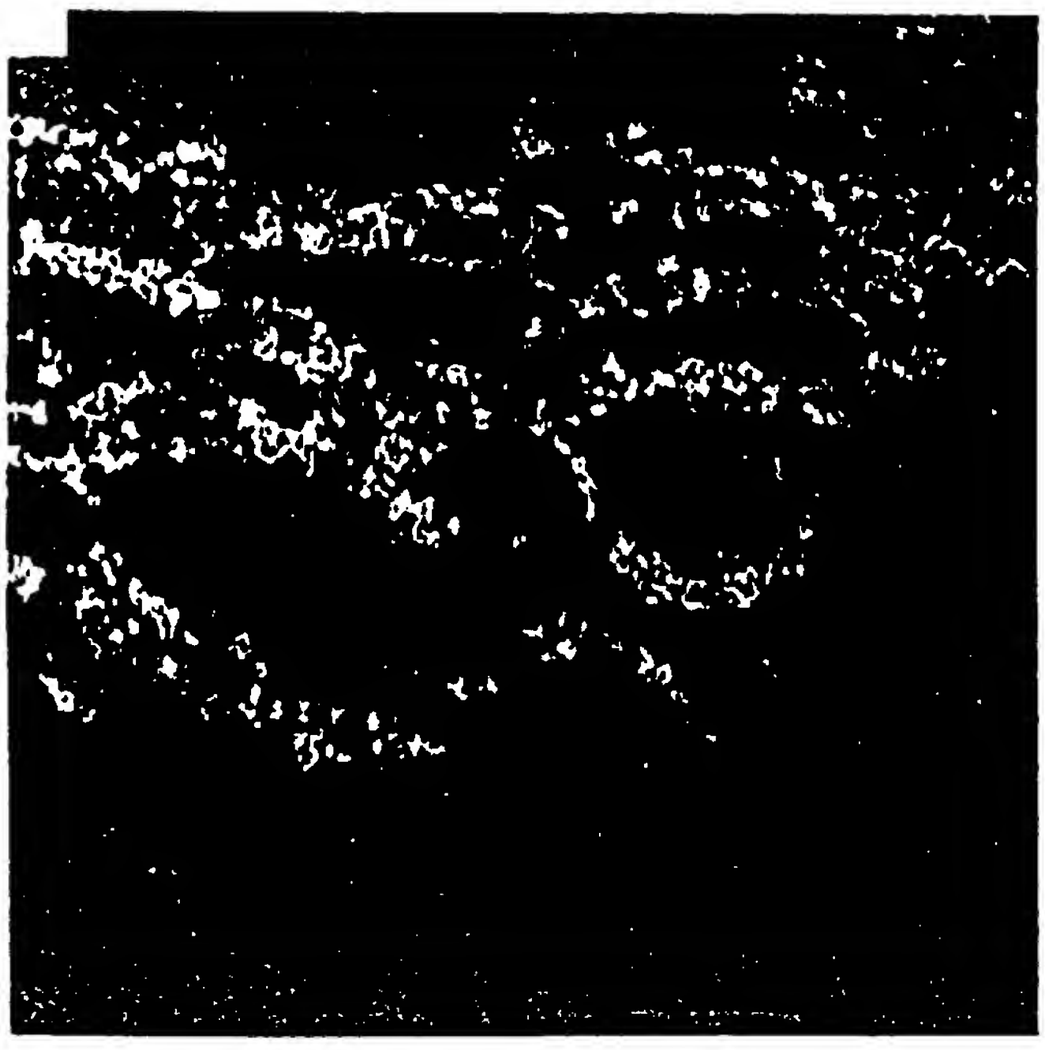


FIG. 8B

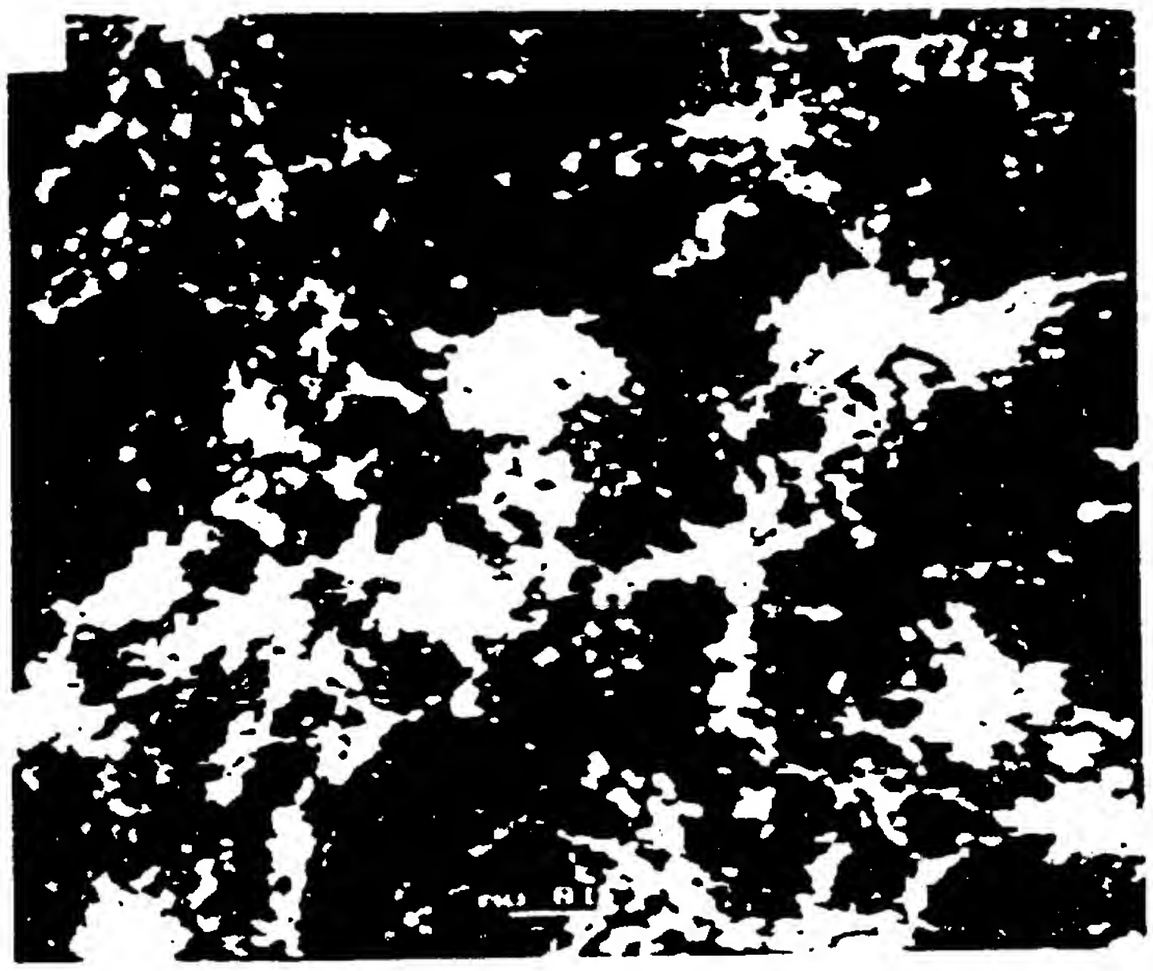


FIG. 9A

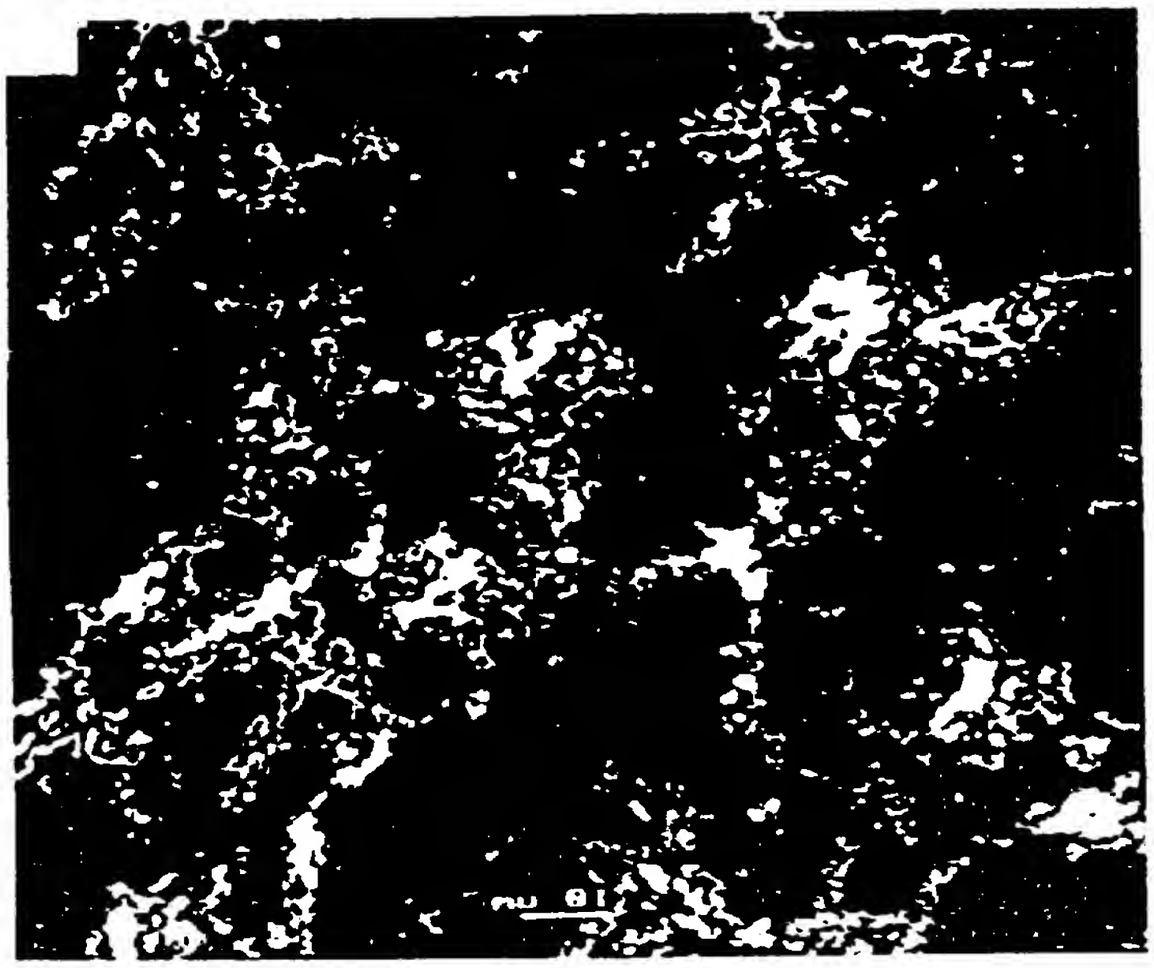


FIG. 9B

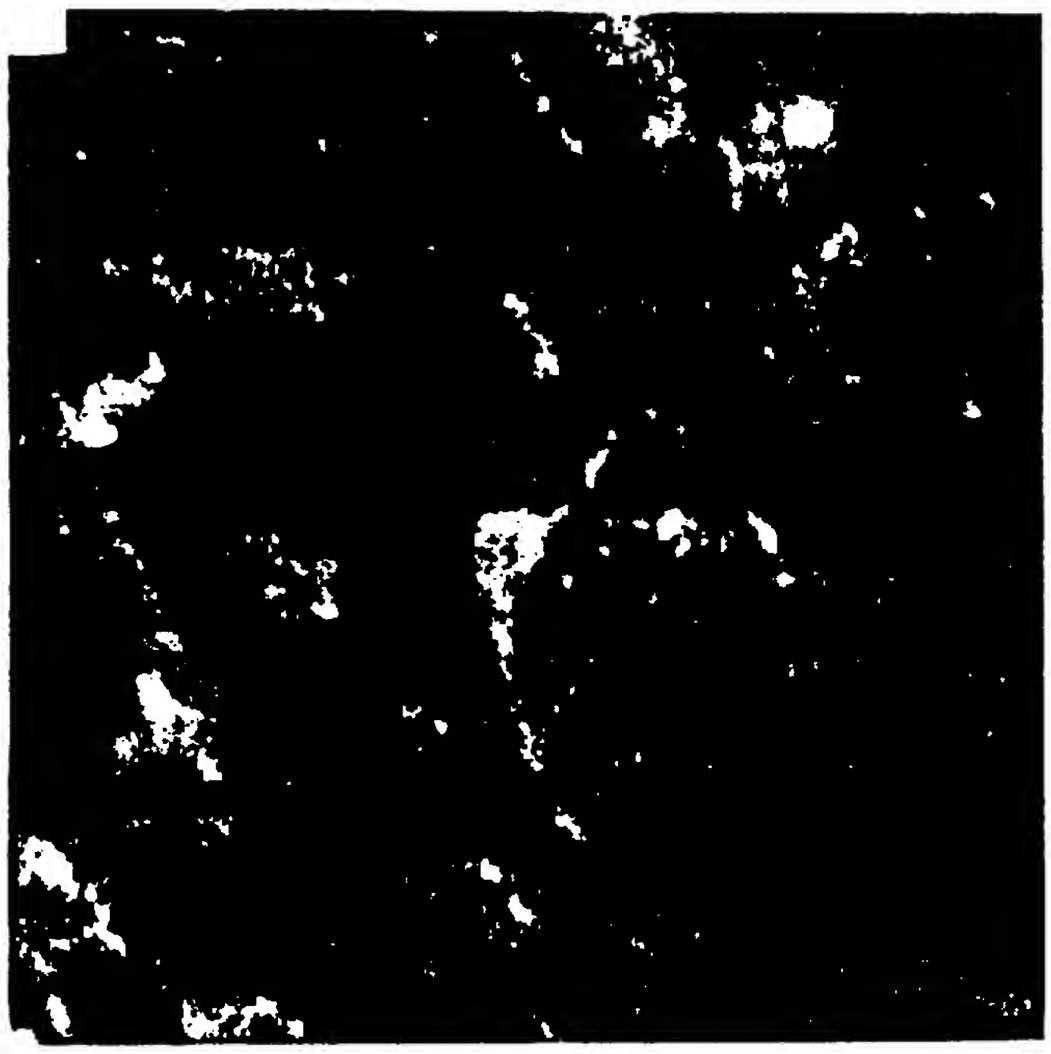


FIG. 10A

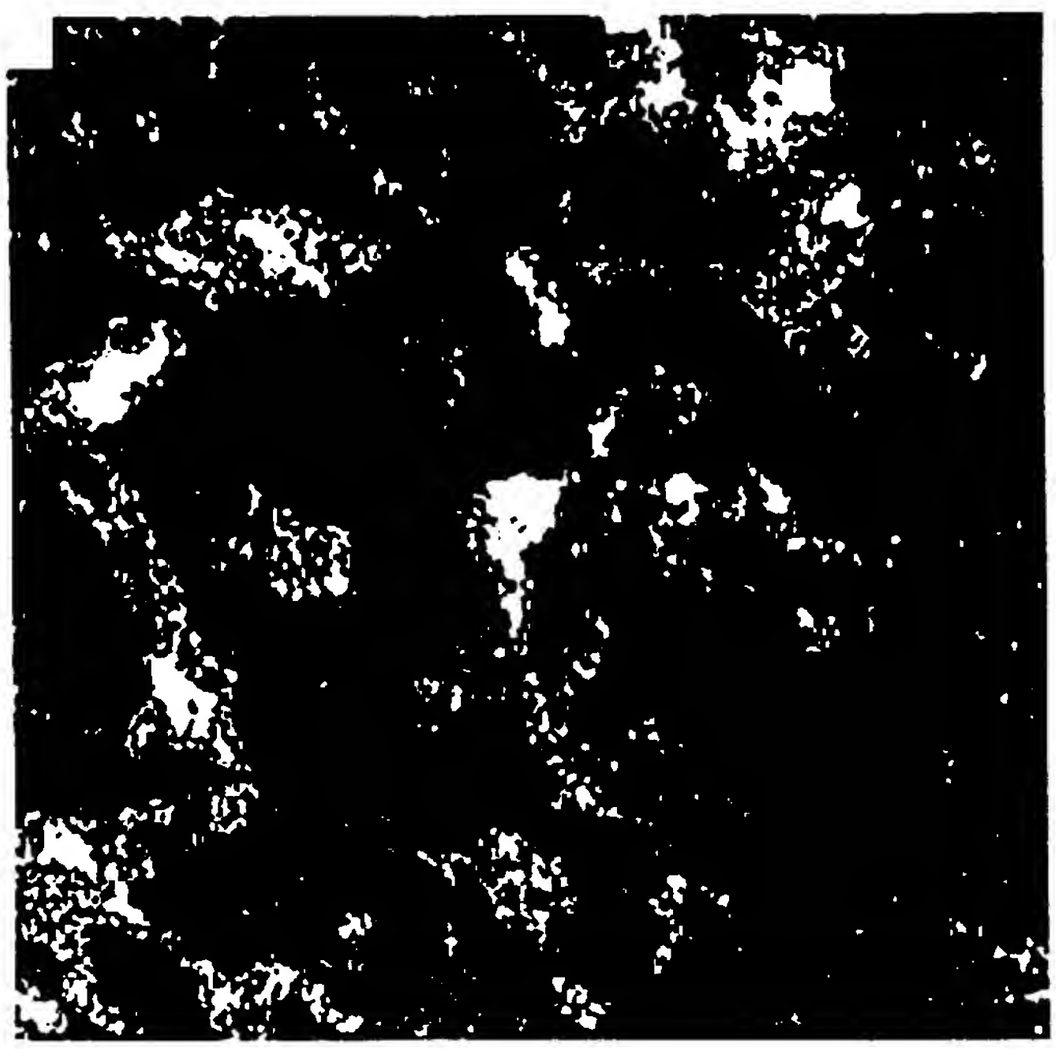


FIG. 10B